THE TEMPERATURE-COEFFICIENTS OF RIBONUCLEASES FROM TWO SPECIES OF GASTROPOD MOLLUSCS FROM DIFFERENT THERMAL ENVIRONMENTS

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Little is known about adaptive differences between temperature-coefficients or activation energies of the enzymes of animals from different thermal environments. This paper supplements our meager knowledge in this field.

The most recent work concerning adaptation at the level of activation energies includes that of Vroman and Brown (1963), Mutchmor and Richards (1961), Lin and Richards (1956) and Kenney and Richards (1955). Richards and co-workers have concerned themselves with arthropod apyrase; they have questioned much of the earlier work on this enzyme on the basis of chemical reasoning (Mutchmor and Richards, 1961). Mutchmor and Richards (1961) have shown that the lower the chill-coma temperature of an arthropod, the higher the activation energy of its muscle apyrase; this may be adaptive since, for arthropods that normally live close to their chill-coma temperatures, an increase in temperature only slightly above that of chill-coma may result in apyrase activity in excess of the minimum required to support muscular function; forms living well above chill-coma temperatures have little need of such an adaptation. On the other hand, Lin and Richards (1956) failed to detect any differences between the activation energies of "proteinase" and invertase from two species of insects with different chill-coma temperatures. The work of Vroman and Brown (1963) on rat and frog liver succinic dehydrogenase contrasts with that of Richards and co-workers but, as suggested by Read (1964), Vroman and Brown neglected their data for the lowest temperature tested in their frog preparation; these authors might otherwise have been led to a conclusion similar to that of Richards and co-workers for apyrase.

In this work the activation energies of ribonuclease components isolated by chromatography from the digestive glands of the gastropods, *Buccinum undatum* L., a form limited to boreal seas, and *Fasciolaria tulipa* L., a species limited to warm temperate to tropical seas, are studied. The two species have similar modes of life, both being scavenging carnivores; they belong to the same super-family, *i.e.*, Buccinacea. The report also provides data concerning molluscan ribonuclease which has previously been studied in squid by Roth and co-workers (Roth and Bachmurski, 1957; Roth, 1959; Edmunds and Roth, 1960).

Materials and Methods

General

Buccinum undatum L. was collected in June in the Salt Pond, Blue Hill, Maine, and in Eggemoggin Reach, off High Head, Brooklin, Maine. *Fasciolaria tulipa* L. was collected from Bimini Lagoon, Bimini, Bahama Islands, in April.

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After the animals had been collected their shells were cracked and the digestive glands pulled free of the remainder of the bodies. The digestive glands were dissected from as much gonad and alimentary tract tissue as possible prior to storage at −20° C.; the possibility that the digestive gland enzymes were contaminated by those from other tissues has to be entertained. Stomachs were empty at the time of dissection.

Material obtained in Bimini was transported packed in dry ice; that from Maine was frozen into a large block of ice which was well insulated for the journey to Boston; all material was frozen on arrival.

Assay for ribonuclease activity

Ribonuclease activity was assayed by a modification of the method of Anfinsen *et al.* (1954). Except where otherwise noted, the procedure used was the following: 1 ml. of pH 5, 0.67 ionic strength acetate buffer and 0.5 ml. of enzyme solution were placed in a test tube; at zero time 1.0 ml. of a 0.4% or 1% solution of ribonucleic acid in water was added; the tube contents were then immediately mixed and the tubes placed in a thermostat for periods ranging from 25 minutes for an incubation temperature of 37° C. to 5 hours for an incubation temperature of 5° C. At the end of this time 0.5 ml. of 0.75% uranyl acetate in 25% perchloric acid was added and the tube contents again mixed; the resulting precipitate was removed by centrifugation. A 0.1-ml. aliquot of the supernatant was then diluted to 3.1 ml. with distilled water and its absorbancy read at 260 mμ. Reagent blanks were run concurrently with the samples undergoing assay. The unit of enzyme activity is defined as an increase, $\Delta(\text{O.D.})_{260}$, of one optical density unit over the reagent blank under the standard conditions of the assay; enzyme blanks, incubated without substrate, were also run.

Purification of substrate ribonucleic acid

Commercial grade yeast ribonucleic acid was dissolved in 1 *M* sodium acetate and dialyzed for three days against frequent changes of distilled water. At the end of this time the dialyzed solution was lyophilized. The resulting material gave a reagent blank at 37° C. in pH 5, 0.27 ionic strength, acetate buffer of about 0.065 optical density unit when read against distilled water.

Purification of ribonucleases

The procedure described below was essentially that followed for the purification of the ribonucleases. All operations were carried out in a cold room at 0–4° C.

Approximately 100 g. of tissue were homogenized with three volumes of distilled water in a Waring Blendor at 4° C. The homogenate was brought to about pH 4 by the addition of glacial acetic acid and centrifuged at 15,000 *g* for 10 minutes. The clarified solution at pH 4 was saturated with ammonium sulfate (75 g./100 ml.), allowed to stand for approximately an hour and centrifuged. The supernatant was discarded and the residue resuspended in distilled water; this suspension was dialyzed for about 20 hours against pH 5, 0.067 ionic strength, acetate buffer. The dialyzed solution was assayed over a wide range of pH before

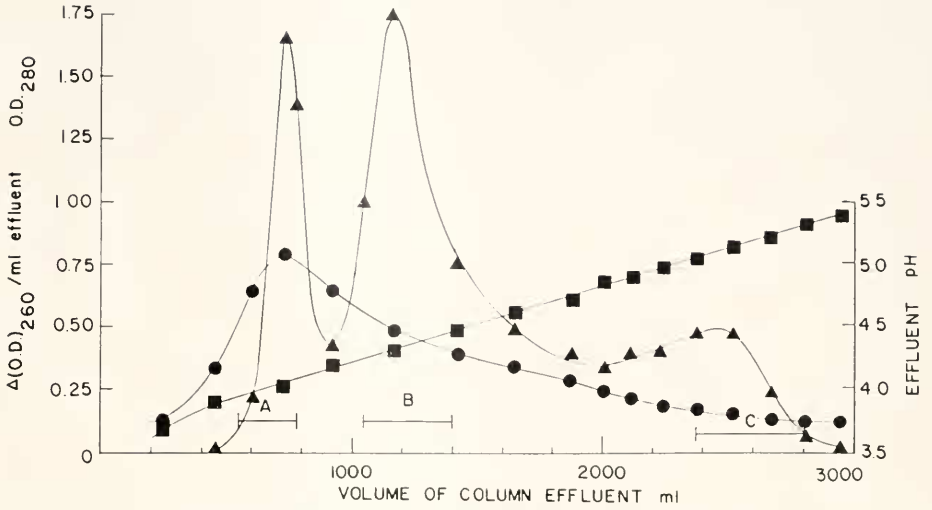

FIGURE 1. Second chromatography of *Buccinum undatum* preparation from 77 g. tissue. Ribonuclease activity of column eluate at pH 5 ▲; OD_{280} of eluate ●; pH of eluate at 5° C. ■. Varigrade elution system: 1 l. each of the following citric acid-sodium citrate buffers: 0.2 *M*, pH 3.9; 0.2 *M*, pH 5.0; 0.2 *M*, pH 6.0; 0.5 *M* pure sodium citrate. Assay temperature: 37° C.

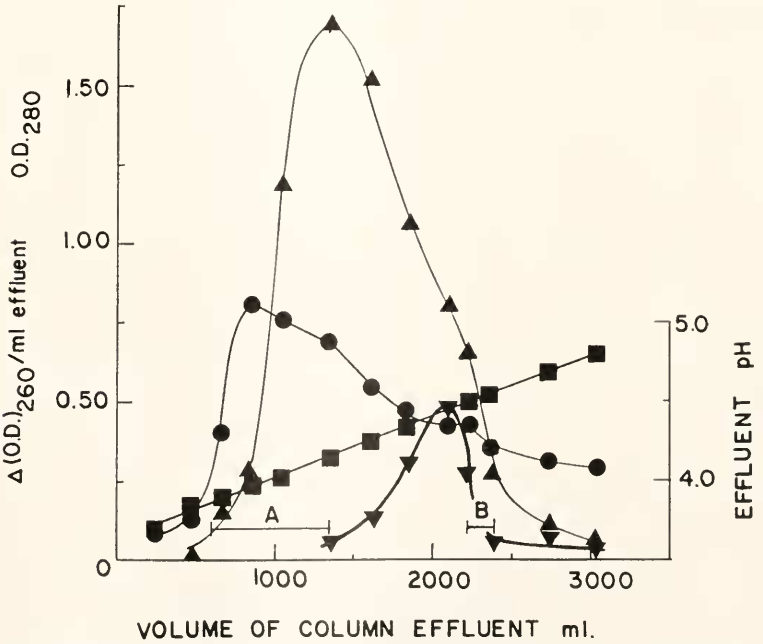

FIGURE 2. Second chromatography of *Fasciolaria tulipa* preparation from 102 g. tissue. Symbols as for Figure 1; ribonuclease activity at pH 7.25 ▼. Varigrade elution system: 1 l. each of the following citric acid-sodium citrate buffers: 0.2 *M*, pH 3.8; 0.2 *M*, pH 4.6; 0.2 *M*, pH 5.2; 0.2 *M*, pH 6.0. Assay temperature: 37° C.

proceeding with the next step involving chromatography on phosphocellulose. To effect this, the dialyzed solution was brought to a pH ranging between 3.2 and 4.0 and filtered through washed phosphocellulose adjusted to the same pH. The column was then washed with a few liters of 0.05 *M* (in citric acid-sodium citrate

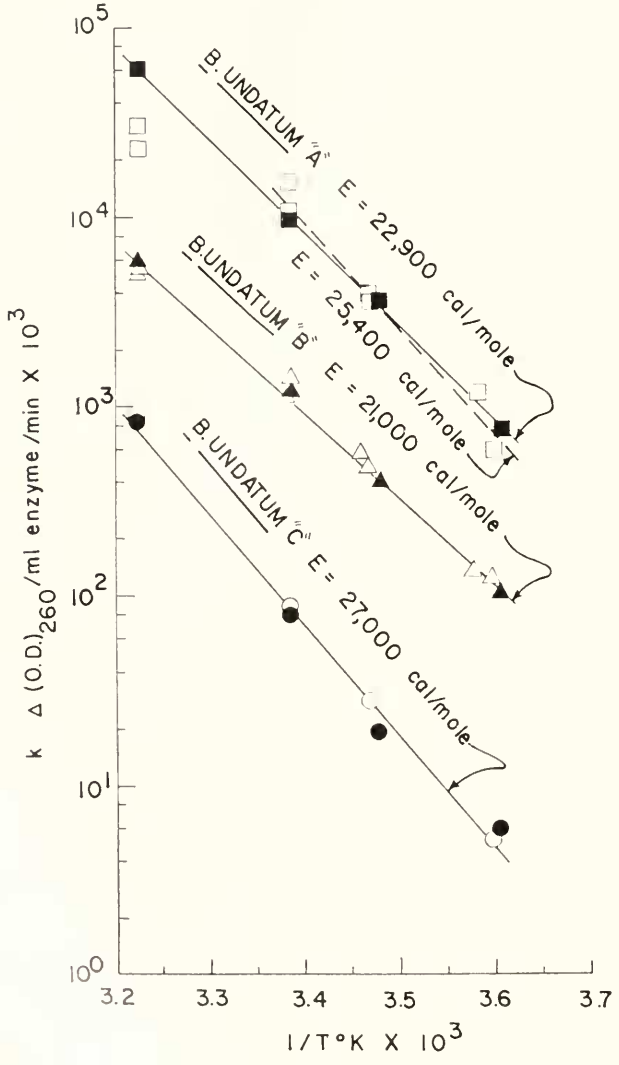

FIGURE 3. Arrhenius plot of activity of ribonuclease A, B and C components of *Buccinum undatum*. Component A ■ □; component B ▲ △; component C ● ○. Closed symbols indicate 1% ribonucleic acid, open symbols 0.4% ribonucleic acid. Divide ordinate by 10 for activity of B component, by 100 for that of A component. Activation energy of 22,900 cal./mole computed from data at 1% substrate concentration over temperature range 5–37° C. ($1/T \times 10^3 = 3.6 - 3.2$). Activation energy of 25,400 cal./mole computed from data at 0.4% and 1% substrate concentration over temperature range 5–22.5° C. ($1/T \times 10^3 = 3.6 - 3.4$).

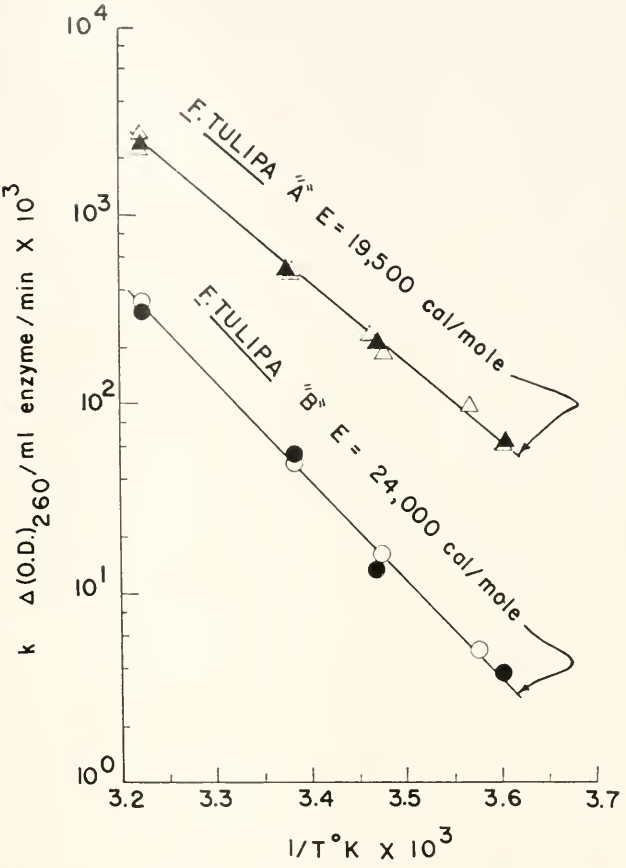

FIGURE 4. Arrhenius plot of activity of ribonuclease A and B components of *Fasciolaria tulipa;* component A ▲ △; component B ● ○; closed and open symbols as described for Figure 3. Divide ordinate by 10 for activity of A component.

combined) citrate buffer of pH 3.2–4.0. After being washed the column was eluted using a four-compartment varigrade apparatus and 0.2 *M* citrate buffers ranging in pH from 3.8 to that of pure sodium citrate. Most of the ribonuclease activity was recovered in the effluent.

After elution the resulting fractions containing ribonuclease activity were pooled, lyophilized, redissolved in distilled water, dialyzed against 0.067 ionic strength, pH 5, acetate buffer and rechromatographed on phosphocellulose. The resulting fractions were dialyzed against 0.2 *M* ammonium bicarbonate, lyophilized and stored at −20° C.

RESULTS

Figures 1 and 2 indicate the results of the chromatography of the digestive gland preparations. *Buccinum undatum* gives rise to three components of ribonuclease activity, *Fasciolaria tulipa* to two. All components exhibited maximal activity

in acetate buffer in the neighborhood of pH 5. A preparation from *Busycon canaliculatum* was also tested and gave an optimum at about pH 5; the drop-off in activity on either side of the optimum was not due to irreversible inactivation since the preparation was stable at 37° C. in pH 3 and pH 8 buffers for an interval equal to that of the incubation period. On the other hand, preparations from *Livona pica* L., *Strombus gigas* L. and *Cassis tuberosa* L. gave optima at about pH 4; *Cassis tuberosa* also gave an additional optimum at about pH 7.

The *Fasciolaria tulipa* preparation gave rise to two non-superposable major peaks when ribonuclease activity was assayed at pH 5 in acetate and 7.25 in trishydroxymethylaminomethane-HCl buffers; in Figure 2 the bump in the pH 5 curve coincides with the peak at pH 7.25. This indicates that the pH 5 peak is composed of at least two different ribonuclease components.

Enzyme kinetics

Arrhenius plots for the several *Buccinum undatum* and *Fasciolaria tulipa* components of ribonuclease activity at pH 5 in 0.27 ionic strength acetate buffer are shown in Figures 3 and 4. Activation energies associated with the *Buccinum undatum* A, B and C components are 22,900 or 25,400 (depending on the data

TABLE I

Statistical limits of confidence in differences between activation energies associated with various components

	Buccinum undatum A *	*Buccinum undatum* A †	*Buccinum undatum* B	*Buccinum undatum* C	*Fasciolaria tulipa* A	*Fasciolaria tulipa* B
Buccinum undatum A *						
Buccinum undatum A †	$0.90 < P < 0.95$	—				
Buccinum undatum B	—	—	—			
Buccinum undatum C	—	—	—	—		
Fasciolaria tulipa A	$0.999 < P$	$0.999 < P$	$0.98 < P < 0.99$	—	—	
Fasciolaria tulipa B			—	$0.99 < P < 0.999$	—	—

* Computed from data obtained with 1% substrate concentration over the temperature range 5–37° C. See Figure 3.

† Computed from data obtained with 0.4% and 1% substrate concentration over the temperature range 5–22.5° C. See Figure 3.

used), 21,000 and 27,000 cal./mole, respectively; for *Fasciolaria tulipa* the corresponding values are 19,500 for the A component and 24,000 cal./mole for the B component.

Values for reaction rates, k, were obtained by extrapolating plots of the extent of reaction *vs.* concentration of enzyme to zero enzyme concentration; the tangents at the origin divided by the times of incubation, which ranged from 25 minutes at 37° C. to 5 hours at 5° C., were taken as the reaction velocities for use in the Arrhenius plots. The *Buccinum undatum* A component gave rise to pronounced downward curvature in plots of enzyme activity *vs.* enzyme concentration at 22.5° C. and 37° C. at 0.4% substrate concentration; an accurate determination of the tangent was thus difficult; at 1% substrate concentration the downward curvature was less apparent and the tangent at the origin could be estimated with greater precision. The other preparations gave more or less linear plots, especially at the lower enzyme concentrations.

When dissolved in buffer all preparations were stable through cycles of freezing, thawing and warming to room temperature, as indicated by the close grouping of the points at each temperature in Figures 3 and 4. These points represent determinations conducted on different days with the same alternately thawed and frozen solution of enzyme. All preparations were stable at 37° C. and pH 5 for 25 minutes.

Slopes of the lines in the Arrhenius plots were computed from the data by the method of least squares and statistical limits of confidence for their differences are given in Table I. The *Buccinum undatum* components as a group have slightly higher activation energies than those of *Fasciolaria tulipa*.

Discussion

Chromatography

The interpretation of the results of the ion-exchange chromatography of proteins should be approached with caution. The presence of multiple peaks of a given type of enzymatic activity may merely indicate that the enzyme in question has given rise to a number of preparative artifacts; evidence for these has recently been treated in a review by Kaplan (1963).

pH optimum

All preparations studied had pH optima in the neighborhood of pH 4–5; these are somewhat lower than the pH optima of mammalian acid ribonucleases. For the bovine pancreatic enzyme Kalnitsky *et al.* (1959) found an optimum at about pH 6.2 in 0.43 ionic strength buffer. De Lamirande and Allard (1959) obtained optima at pH 5.8 for rat liver enzyme and pH 5.5 for rat brain enzyme. Maver *et al.* (1959) found similar values for calf spleen ribonuclease. For heated squid caecal fluid enzyme, Edmonds and Roth (1960) demonstrated activity through the range pH 4–7 with a peak at about pH 5.2. Similar results were obtained for a squid gill preparation.

Enzyme kinetics

The determination of the activation energies of the complex reactions involved in this work is fraught with uncertainties. The first of these involves changes in

the Michaelis constant, K_m, of the enzymes with temperature; if these are not taken into account, erroneous values of V may result (Dixon and Webb, 1958). This complication can be avoided by determining the activation energies associated with each enzyme at two different substrate concentrations; if identical Arrhenius plots are so obtained, the enzymes can be considered saturated with substrate at each temperature. The second complication is inherent in the complexity of the substrate molecule. Thus, the enzyme reacts with the original substrate molecule producing fragments, probably of variable composition, that decrease in size with time. These may be subsequently attacked by the enzyme at different rates so that the most labile are broken down first, resulting in the enrichment of the reaction mixture with nucleotide moieties less susceptible to attack and possibly inhibiting. In view of these complications it would again seem reasonable that they could be disregarded if the reaction velocities could be proven to be independent of substrate concentration.

Accordingly, the enzyme components were assayed at two substrate concentrations, 0.4% and 1%, to clarify the possible effects of the variables just discussed. Except for the *Buccinum undatum* A component, the rate of reaction appears not to be dependent on substrate concentration and even this exception could possibly be ascribed to chance alone, as judged by the scatter of the data in Figure 3.

Values of activation energies associated with the *Buccinum undatum* A and B components and the *Fasciolaria tulipa* A component only approximate that obtained by Kalnitsky *et al.* (1959) for bovine pancreatic ribonuclease between 30 and 50° C., *i.e.*, 20,290 cal./mole.

The chromatograms for the *Buccinum undatum* and *Fasciolaria tulipa* preparations in Figures 1 and 2, respectively, indicate that the *Buccinum undatum* A, B and C, and *Fasciolaria tulipa* A components are relatively "pure" with respect to contamination with other active components; however, the possibility must be entertained that the *Fasciolaria tulipa* B component is contaminated by the *Fasciolaria tulipa* A component. This would result in a concomitant lowering of the activation energy of the *Fasciolaria tulipa* B preparation.

Ecology and comparative physiology of the species investigated

Buccinum undatum ranges from Labrador to New Jersey (Johnson, 1934) and is adapted to a cool environment. Gowanloch (1927) has exposed individuals of this species to increases of temperature of 1° C. per 5-minute interval and noted that under these conditions death occurs at 29° C.; this suggests that in nature the species would be able to survive only at temperatures well below this value.

Fasciolaria tulipa is a warm-water species and ranges from North Carolina to Florida, Texas (Johnson, 1934) and the West Indies (Warmke and Abbott, 1961). At Bimini it is found half submerged, crawling about over the exposed flats in the lagoon at low tide. During the summer the water temperature of the tide pools on the flats probably rises into the mid-thirties since surface water temperatures of the Gulf Stream near Bimini reach almost 29° C. at this time (Fuglister, 1947). *Fasciolaria tulipa* thus normally lives in a temperature range well above that lethal to *Buccinum undatum;* the enzymes of the two species might therefore be expected to be adapted accordingly.

Conclusions

The activation energies of the *Buccinum undatum* components as a group are slightly higher than those of *Fasciolaria tulipa*. Their range of variation does not approach that found by Mutchmor and Richards (1961) for arthropod apyrase; here activation energies ranging from 11,600 to 25,600 cal./mole were found and these data fit in well with the rationale of adaptation proposed by the authors. It is therefore concluded that in contrast to the apyrase of arthropods or vertebrate succinic dehydrogenase (Read, 1964), adaptation of gastropod digestive gland ribonuclease by way of variation of activation energy is either nugatory or, like that of the insect digestive enzymes described by Lin and Richards (1956), non-existent.

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Note Added in Proof

Since this article went to press Licht (*Comp. Biochem. Physiol.*, 13: 27–34, 1964; *ibid*, 12: 331–340, 1964) has reported data relative to muscular contraction and temperature coefficients of alkaline phosphatase and ATPase in reptiles from different thermal environments; in addition, Baslow and Nigrelli (*Zoologica N. Y.*, 49, 41–51, 1964) have suggested that there are differences in Q_{10} as a function of temperature in the brain cholinesterase of cold- and warm-water fish.

Summary

1. *Buccinum undatum* digestive gland ribonuclease chromatographed on phosphocellulose consists of three enzymatic components, all showing an optimum pH of about pH 5 in acetate buffer. Activation energies of the three components are 21,000, 22,900 and 27,000 cal./mole at pH 5.

2. *Fasciolaria tulipa* digestive gland ribonuclease chromatographed on phosphocellulose consists of two enzymatic components, both with pH optima near pH 5 in acetate buffer. Activation energies of the two components are 19,500 and 24,000 cal./mole at pH 5.

3. It is concluded that the ribonucleases of gastropod digestive glands show little or no adaptation, by way of differences in activation energies, to the thermal environment.

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